New γ-L-Glutamyl Peptides in Onion (*Allium cepa*). III

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The investigation of γ-glutamyl peptides in onion has been continued and a number of new peptides have been isolated in crystalline or chromatographically pure form. With one exception their structures have also been established. All the known γ-glutamyl peptides in onion are arranged below in the order in which they are eluted with acids from a Dowex 1 column:

1. γ-L-Glutamyl-L-valine, crystalline, white needles
2. γ-L-Glutamyl-L-isoleucine, crystalline, white needles
3. γ-Glutamyl-leucine, crystalline
4. γ-L-Glutamyl-cysteine derivative, structure not known, amorphous, white powder, gradually turning yellow at room temperature
5. Ethyl ester of γ-L-glutamyl-S-[β-carboxy-n-propyl]-L-cysteinyl-glycine, crystalline
6. γ-L-Glutamyl-methionine, crystalline, white plates
7. γ-Glutamyl-S-methyl cysteine, crystalline
8. γ-L-Glutamyl-L-phenylalanine, crystalline, white needles
9. γ-L-Glutamyl-S-[β-carboxy-n-propyl]-L-cysteinyl-glycine, white needles.

Of these peptides the last two (8 and 9) have been described in detail in earlier papers[1,2]. Of the other peptides in the list the ethyl ester of the previously isolated γ-L-glutamyl-S-[β-carboxy-n-propyl]-L-cysteinyl-glycine (5) was possibly formed in the Amberlite IR-120 column during the isolation. The esterified peptide may therefore be an artefact and not present in the onion. This is not certain, however.

The sulphur-containing γ-glutamyl peptide (4), which was briefly mentioned in an earlier paper and the composition of which was $C_{11}H_{20}N_2O_7S$ according to the first elementary analysis, is a rather unstable compound which gradually turns yellow at room temperature. The elementary composition of the compound is therefore uncertain. The instability is due to the sulphur-containing amino acid present in it. This amino acid decomposes readily when the peptide is hydrolyzed and could not therefore be isolated. The amino acid is a cysteine derivative because glutamic acid and cysteine/cystine are formed when the peptide is hydrolyzed with 6 N HCl. γ-Glutamyl-alanine, glutamic acid, and alanine are formed after its treatment with Raney nickel in boiling 90 % methanol and subsequent mild hydrolysis. This peptide is present in the onion in a larger amount (about 1.3 % of the dry matter) than any other γ-glutamyl peptide. The elucidation of the structure of this peptide is being continued.

If the above-mentioned ethyl ester is an artefact, eight different γ-glutamyl peptides have thus been isolated from the onion. Peptides 3 and 7 were isolated in amounts so small that the amino acids formed on acid hydrolysis could be characterized only by paper chromatography. The optical rotations could not therefore be measured. All the other peptides were characterized by elementary analysis, isolation of the amino acids formed on hydrolysis and detailed studies of these amino acids.

The number of γ-glutamyl peptides in onion is appreciably larger than the eight isolated so far in pure form. This is seen in Fig. 1 which shows the fractionation of the ninhydrin-positive substances in onion in a Dowex 1 column after the amino acids had been eluted.

Five kilograms of crushed onions were thoroughly extracted with about 80 % ethanol at a low temperature and the clear solution obtained was passed through an Amberlite IR-120 column. The amino acids and peptides remaining in the column were eluted with 1 N ammonia. The residue remaining after the ammonia had been evaporated to dryness in a vacuum was dissolved in 0.5 N acetic acid and fractionated in a Dowex 1 column (7 × 68 cm) with 0.5 N acetic acid. The neutral and basic amino acids, glutamic

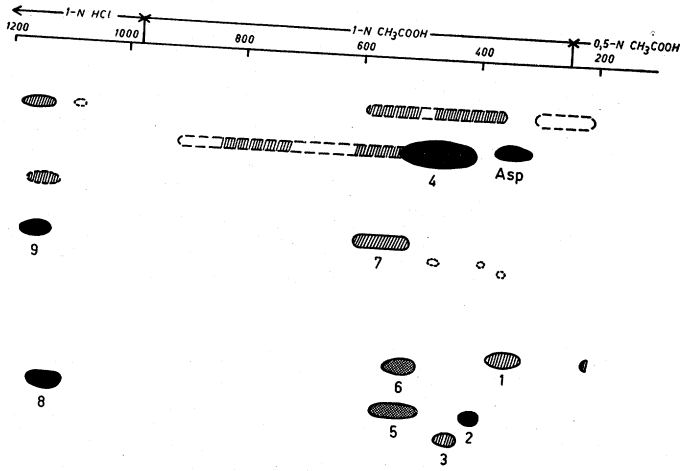

Fig. 1. γ-Glutamyl peptides eluted from a Dowex 1 column by 1 N acetic and hydrochloric acids Those marked with a number have been isolated; their structures are given in the text. Those without numbers have not yet been identified.

acid and most of the tryptophane emerged from the column in the first 200 fractions (à 25 ml). After 250 fractions had been collected, the elution was continued with 1 N acetic acid. From fraction 979 onwards 1 N HCl was used as the eluant. Fraction 1 277 was the last one taken.

The ninhydrin-positive substances in fractions 250—1 200 which contained the γ-glutamyl peptides emerged from the Dowex 1 column as shown in the figure. The location was established by one-dimensional paper chromatography of every second fraction employing butanol-acetic acid-water as solvent.

The isolation of different peptides from the fractions was accomplished in cellulose powder columns with n-butanol-acetic acid-water (63:10:27). The γ-glutamyl peptides were isolated in cellulose powder columns from different Dowex 1 effluent fractions as follows:

γ-Glutamyl-valine from fractions 310—369
γ-Glutamyl-isoleucine from fractions 382—407
γ-Glutamyl-leucine from fractions 408—478
The γ-glutamyl peptide which contained the unstable cysteine derivative mentioned above from fractions 406—540
The ethyl ester of γ-glutamyl peptide 9 from fractions 479—569
γ-Glutamyl-methionine from fractions 479—569
γ-Glutamyl-S-methylcysteine from fractions 510—604
γ-Glutamyl-phenylalanine from fractions 1 090—1 150
γ-Glutamyl-S-[β-carboxy-n-propyl]-cysteinyl-glycine from fractions 1 120—1 175.

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References

1. Virtanen, A. I. and Matikkala, E. J. *Suomen Kemistilehti B* **33** (1960) 83.
2. Virtanen, A. I. and Matikkala, E. J. *Hoppe-Seyler's Z. physiol. Chem.* **322** (1960) 8.

weight being offered. They were basicified to pH 4.0 with sodium bicarbonate. Following tanning they were given a short wash, but they were not neutralized. The butt for retanning was drummed in a 70° Bk. vegetable tan liquor (*see below*), tan being added until penetration was almost complete.

The butts for vegetable and semichrome tannage were tanned on the countercurrent system with a blend of lightly sulfited quebracho containing 10% chestnut. The barkometer of the final liquor was about 70°.

The vegetable-tanned butt was given a very short wash. The butt for semichrome leather was washed well and then drummed with the chrome liquor described above, 2% Cr_2O_3 on limed weight being offered.

The leathers were then air-sammed, lightly oiled with neatsfoot oil, nailed on frames, and dried at atmospheric temperature. The analyses of the leathers are given in Table I. The chrome leather had a higher grease content than intended, presumably because the original hide had a high natural fat content.

TABLE I

ANALYSIS OF LEATHERS BEFORE STORAGE

(*Grams/100 grams air-dry leather*)

	Chrome	Vegetable	Semichrome	Chrome Retan
Moisture	15.6	12.4	14.6	12.5
Grease	8.3	2.9	3.2	3.7
Water solubles	5.6	17.6	3.3	8.3
Insoluble ash	4.3	0.2	2.8	1.8
Hide substance	63.7	43.9	52.8	51.4
Cr_2O_3	3.9	—	2.4	1.8
Fixed tans*	—	23.0	20.0	19.7
Degree of tannage	—	52	38	38
pH of water extract	3.9	4.2	3.3	3.2

*Fixed tans were determined by difference using a factor of 2.5 × Cr_2O_3 for "chrome tan" in the combination-tanned leathers.

Method of storage.—The leathers were stored at approximately 100% R.H. at 60°C. for 2, 5, 10, or 15 weeks and at 40°C. for 12, 36, and 72 weeks. They were hung from glass rods over water in closed jars, which were then placed in incubators at the appropriate temperatures. Duplicate sets were stored at 40°C. with a small beaker of toluene in the bottom of the jar to discourage mold growth. This was effective provided the slow loss of toluene by evaporation was made good when necessary. The control samples were stored in the laboratory (18°–22°C.).

Sampling.—Buckle tear rather than tensile strength determinations were used to assess losses in strength, since the samples required are smaller

and more compact. A preliminary test indicated that eight samples for each period of storage would give an adequate estimate of the changes in strength.

The pieces of leather were cut as indicated in Fig. 1. The butt was divided into sixteen blocks of nine pieces cut symmetrically with respect to the backbone. Pieces from each pair of symmetrically placed blocks were then allotted at random to the treatments in such a way that each experimental piece could be compared with a corresponding control piece cut from the opposite side of the backbone.

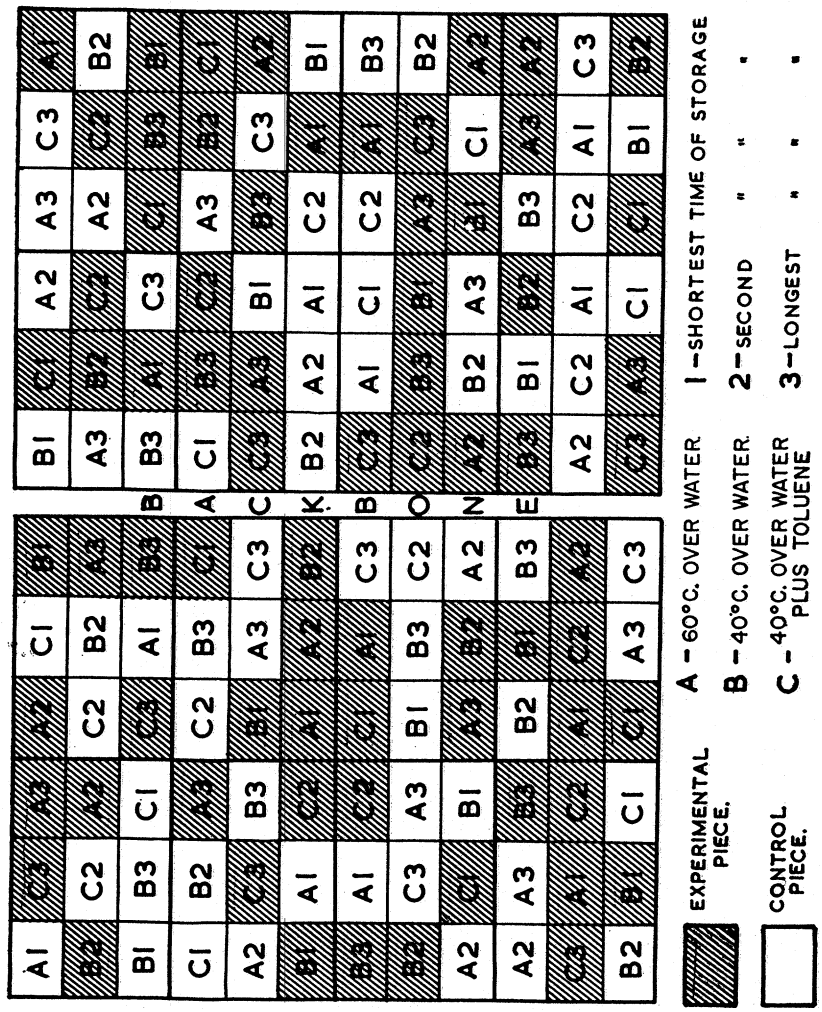


FIGURE 1.—Typical sample-cutting plan. Each experimental piece is paired with a control piece cut symmetrically from the opposite side of the backbone.

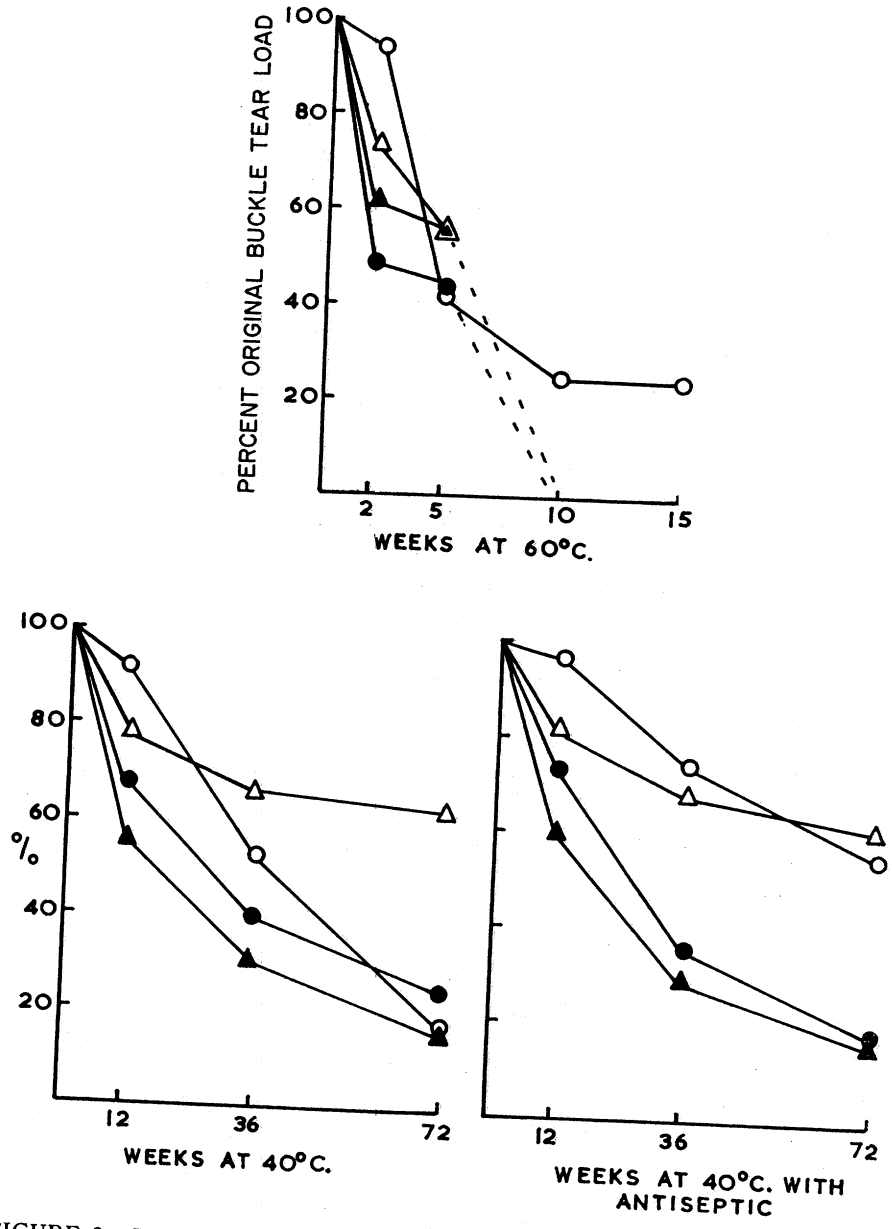


FIGURE 2.—Percentage of original buckle tear load remaining after storage.

△ Vegetable-tanned
○ Chrome-tanned
▲ Semichrome
● Chrome retan

Buckle tear load.—The buckle tear load was determined by the S.L.T.C. official method (12) except that the pieces were 3½" x 1½". Each control piece was tested at the same time as its experimental partner.

Shrinkage temperature.—The determinations on the vegetable-tanned leather were made in water, and on the other three leathers in 75% v/v glycerol/water. All samples were thoroughly wet back with water before testing. The results are the mean of at least three determinations.

Analysis of leather.—The leathers were cut into about 3-mm. squares, or if too brittle to cut, they were ground to pass a 20-mesh sieve.

Analyses were then carried out as described in Part I.

Extractions with 0.1*N* $NaHCO_3$, 2*M* $CaCl_2$, and 0.1*M* acetic acid were made using a 1:10 w/v ratio of leather to solution for 48 hours at room temperature with occasional shaking. Extractions with the same ratio of 5*M* acetic acid were for 7 days at room temperature, as it was found that the extraction was slower than in the other solutions.

The total nitrogen in the extracts was determined by a standard micro-Kjeldahl procedure (13) and the volatile nitrogen by direct distillation from borate buffer at pH 11, after neutralization with sodium hydroxide where necessary.

With the chrome, semichrome, and chrome retan leathers the chrome content of the 5*M* acetic acid extracts was determined by the perchloric acid oxidation method.

RESULTS

Appearance and loss in strength.—After only two weeks' storage over water at 60°C. all the leathers containing vegetable tan had darkened in color. The semichrome and chrome retan leathers had lost 40% to 50% of their original strength and the vegetable-tanned leather 26%, but the chrome leather was unaffected.

After five weeks, however, the difference between the leathers was less, since further losses in strength of the semichrome and chrome retan leathers were small, while that of the chrome leather was considerable.

On further storage the vegetable-tanned leather deteriorated rapidly; after ten weeks it had lost its fiber structure and was dripping as a very dark viscous fluid on the bottom of the jar. It was, therefore, removed from storage. The chrome leather at this stage retained only 25% of its original strength.

After a further five-week storage the combination-tanned leathers had also lost their fiber structure, were very dark in color, and had become toffee-like in consistency. The chrome leather showed little change in color or appearance. The grain was rather cracky, but otherwise it looked to be a

satisfactory piece of leather. Its strength had not been reduced in the last five weeks of storage and remained at 25% of its original value.

Toluene effectively prevented mold growth on the leathers stored at 40°C. In the absence of toluene the three leathers containing vegetable tan rapidly developed a profuse mold growth. The chrome leather remained free of mold for about 12 weeks, and growth was never as extensive as on the other leathers. This leather, however, was the only one whose behavior was appreciably affected by the mold growth (*see Fig. 2*). Losses in strength of the vegetable, semichrome, and chrome retan leathers were similar under both conditions of storage at 40°C., but with the chrome leather losses were much greater in the presence of mold.

The vegetable-tanned leather was much more resistant to storage at 40°C. over water than at 60°C. over water, and after 72 weeks it had only lost about 30% of its strength. It was slightly darker in color than at the start of storage, but otherwise its appearance was still quite good. The semichrome and chrome retan leathers were by far the most adversely affected. After 72 weeks they had lost about 80% of their strength and were beginning to show the plastic flow and loss of fiber structure found after 15 weeks at 60°C.

A microscopical examination of the fiber structure of the leathers confirmed the conclusion drawn from their macroscopic appearance. In the case of the chrome leather stored at 60°C. over water, however, although the fiber structure was still quite good, the fibers had a slightly glassy appearance, and the structure was rather more open than in the control samples.

Shrinkage temperature.—The shrinkage temperature of the chrome leather increased during storage, except when there was mold growth on the leather. The shrinkage temperature of the vegetable-tanned leather also tended to increase. Measurements made on the few pieces which still retained some fiber structure after 10 weeks at 60°C. still indicated a shrinkage temperature of 80°C. The shrinkage temperatures of the combination-tanned leathers decreased steadily as storage proceeded.

pH of water extract.—There was little change in pH of water extract as a result of storage at 60°C. over water. With the vegetable-tanned leather there was a slight decrease in pH, and with the chrome leather there was a slight increase. With the combination-tanned leathers a slight decrease was followed by an increase to values above the initial pH. A similar observation was made by Sykes and Williams-Wynn on accelerated aging tests on chrome retan leathers (11).

At 40°C. in the absence of mold there were small decreases in pH of both chrome- and vegetable-tanned leathers. As at 60°C. the pH of both the semichrome and retan leathers decreased at first, and then increased, but in this case the pH of the retan leather rose above 5.0.

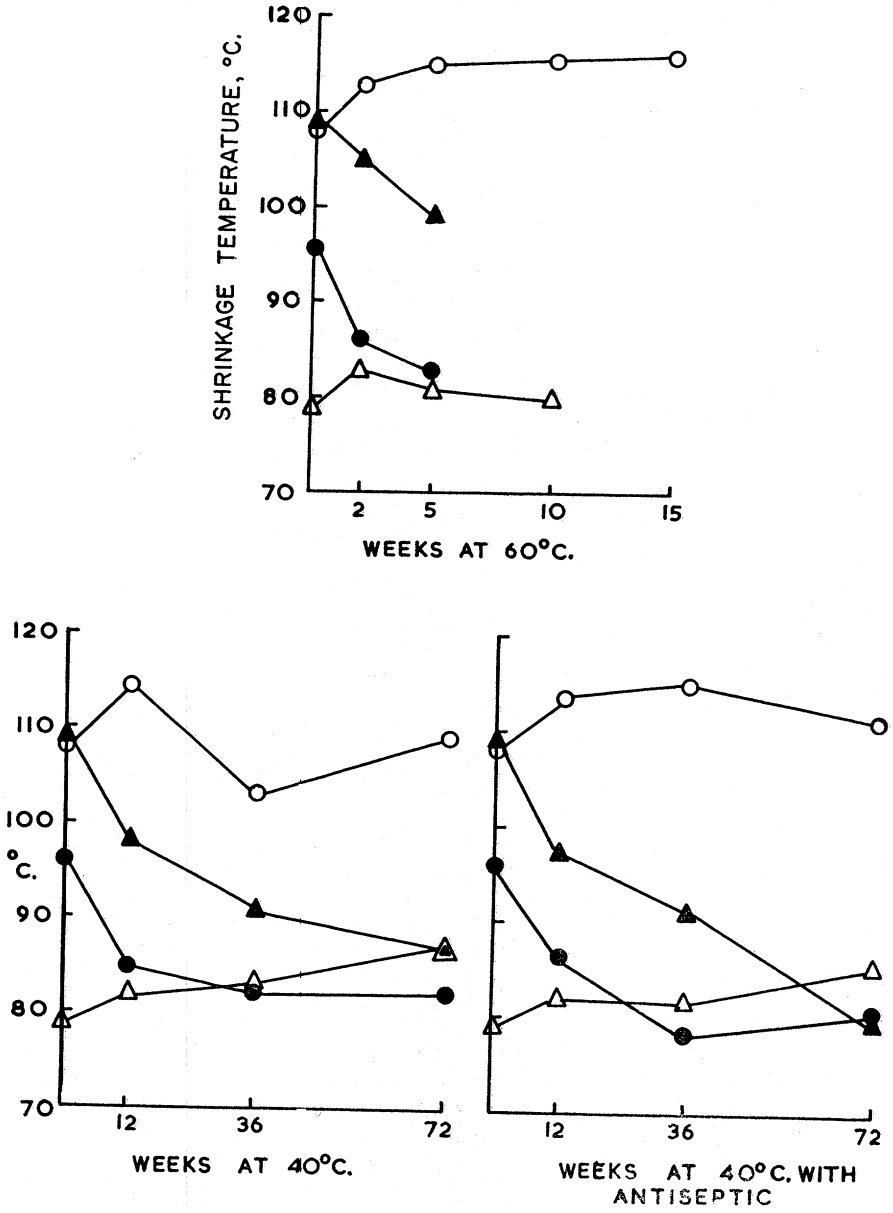


FIGURE 3.—Shrinkage temperatures of leathers after storage. Vegetable-tanned leather measured in water, others in 25% aqueous glycerol.

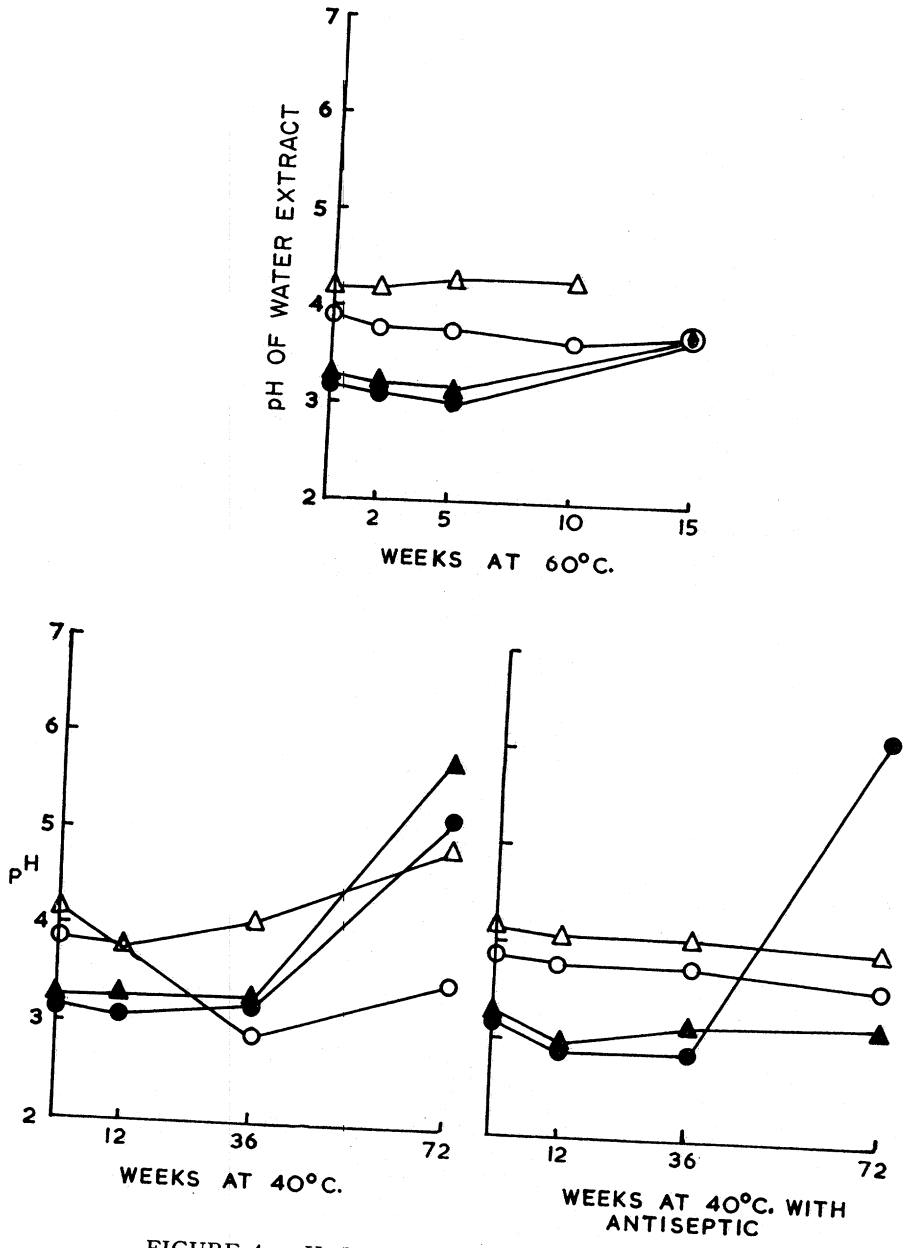


FIGURE 4.—pH of water extract of leathers after storage.

△ Vegetable-tanned
○ Chrome-tanned
▲ Semichrome
● Chrome retan

When mold growth occurred, the pH changes were greater. The vegetable-tanned leather showed a decrease followed by an increase, as did the combination-tanned leathers. The chrome-tanned leather was relatively unaffected after 12 weeks, but further storage produced a large decrease followed by a small increase.

Extraction of degraded protein.—The breakdown of protein in leather has usually been followed by extraction with mildly alkaline solutions and determination of the dissolved protein as nitrogen. The results so obtained have generally shown little correlation with strength changes (3, 5).

In a preliminary examination samples of the vegetable- and chrome-tanned leathers stored at 60 °C. over water for the longest time were extracted with 0.1*N* $NaHCO_3$. The leather was filtered off, and the nitrogen was determined on the extract (*see Table II*). The soluble nitrogen of the deteriorated leathers was only slightly greater than that of the controls. The vegetable-tanned leather had lost its fiber structure completely during storage, and it was evident that these small increases in soluble nitrogen were not a satisfactory indication of the extent of protein breakdown.

The extractions were, therefore, repeated, using 2*M* calcium chloride and 0.1*M* acetic acid, which are better solvents for collagen than 0.1*N* sodium bicarbonate. Although the amounts of nitrogen extracted by both these solutions were rather greater than with bicarbonate, they were still not consistent with the visible degradation.

An attempt was therefore made to bring more degraded protein into solution using 5*M* acetic acid. This extracted over 20% of the nitrogen from the

TABLE II

SOLUBLE NITROGEN FOUND IN EXTRACTS OF CHROME AND VEGETABLE-TANNED LEATHERS

(*Soluble nitrogen as % of total nitrogen*)

Extractant	Chrome		Vegetable	
	Control	Deteriorated 15 weeks at 60°C.	Control	Deteriorated 10 weeks at 60°C.
0.1*N* $NaHCO_3$	0.5	1.5	0.5	1.5
2*M* $CaCl_2$				
nonvolatile	0.06	0.16	0.14	3.37
volatile	0.74	1.58	1.06	1.13
0.1*M* acetic acid				
nonvolatile	0.12	0.24	0.29	2.90
volatile	0.76	1.60	1.07	1.16
5*M* acetic acid				
nonvolatile	0.14	0.98	0.61	23.2
volatile	0.70	1.48	1.11	1.38

vegetable-tanned leather. Further determinations showed that the soluble nitrogen increased with the deterioration. Neutralization of the 5*M* acetic acid extracts precipitated a large proportion of the material. On paper electrophoresis of the extracts some separation was obtained. With the 5*M* acetic acid extracts most of the material stayed at the origin and stained with naphthalene black, while with the 0.1*M* extracts most of the material stained with ninhydrin. It would, therefore, seem that 0.1*M* acetic acid extracts mainly small peptides and amino acids and most of the volatile-nitrogen, while the 5*M* acetic acid, in addition, dissolves quite large protein fragments. The composition of these extracts and of the degraded protein as a whole are being examined. The volatile nitrogen probably consists mainly of ammonia and other simple compounds. It appeared, therefore, that extraction with these two solutions should give useful information on the protein breakdown.

All leathers were therefore extracted with these two solvents, and volatile and total nitrogen were determined (*see Fig. 5.*).

At 60°C. the soluble nitrogen extracted increased slowly with time of storage but only reached high values when degradation was extensive, that is, with the vegetable, semichrome, and retan leathers, with values of 21.2%, 14.1%, and 28.7% respectively. Increases in soluble nitrogen occurred most rapidly with the vegetable-tanned leather. With the chrome leather little was extracted even after storage for 15 weeks, although its strength had been reduced to 25% of the original value. Increases in volatile nitrogen were small except with the combination-tanned leathers.

At 40°C. increases in soluble nitrogen were smaller than at 60°C. although in many instances losses in strength on prolonged storage were similar to, or greater than, those occurring during the shorter periods at the higher temperature. Again, high values were only obtained when the leathers were beginning to show signs of gross deterioration. In the absence of mold both volatile and nonvolatile nitrogen were low in the vegetable- and chrome-tanned leathers, even after 72 weeks. Appreciably greater amounts of nitrogen were extracted from the combination-tanned leathers. Mold growth on the chrome- and vegetable-tanned leathers increased the volatile nitrogen but had little effect on the nonvolatile nitrogen. The total amounts of nitrogen extracted from the semichrome and retan leathers was slightly less in the presence of mold. With the semichrome leather the volatile nitrogen was, however, markedly increased at the expense of the nonvolatile. With the retan leather both volatile and nonvolatile nitrogen were decreased. The volatile nitrogen of the retan leather in the absence of mold was, however, particularly high.

Extraction of chrome tan.—The 5*M* acetic acid extracted about 60% of the chrome from the original chrome leather, but after a short time of storage at either temperature this had dropped to about 45%, and on further

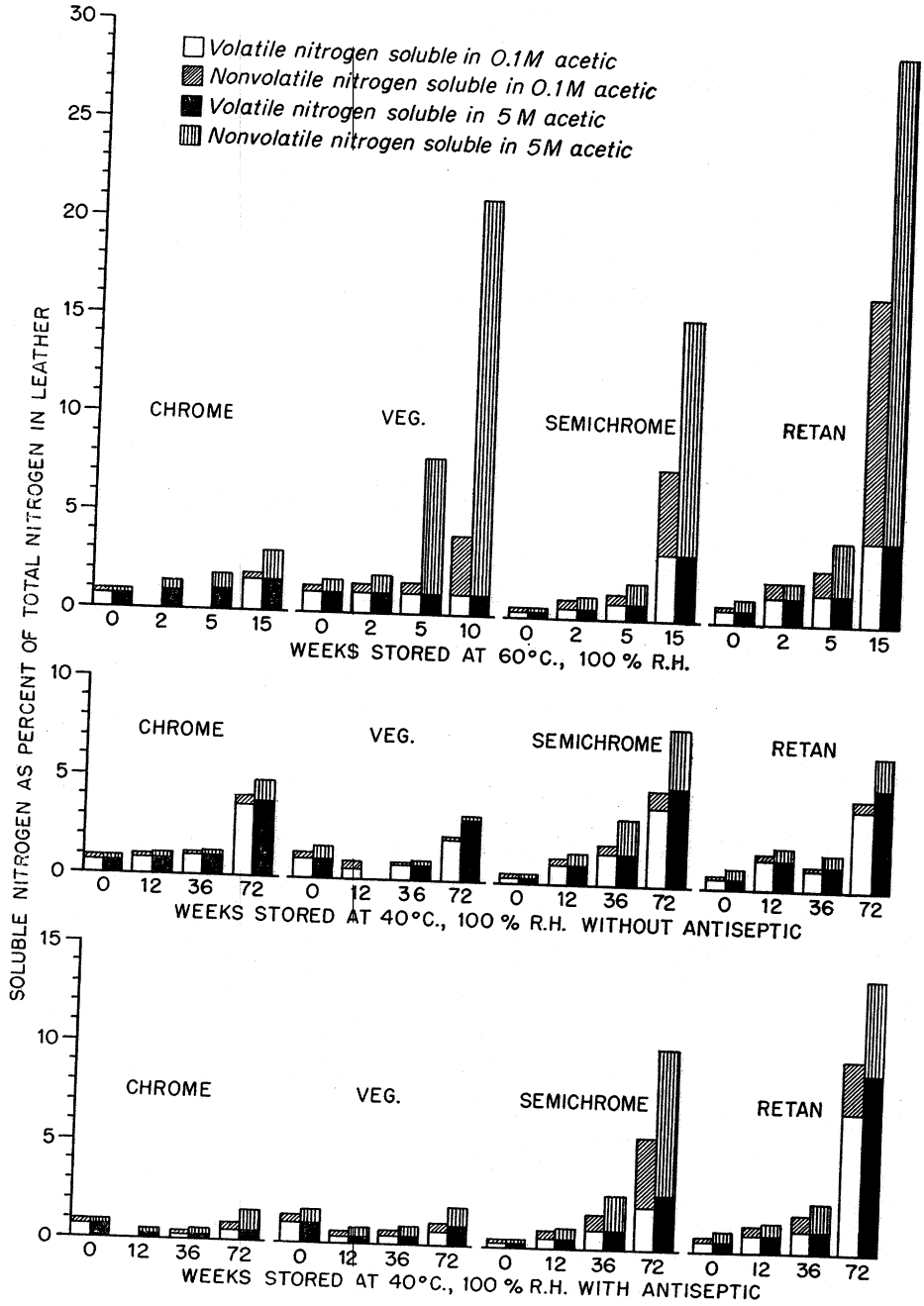


FIGURE 5.—Soluble, volatile, and nonvolatile nitrogen extracted from stored leathers by 0.1*M* acetic acid and 5*M* acetic acid.

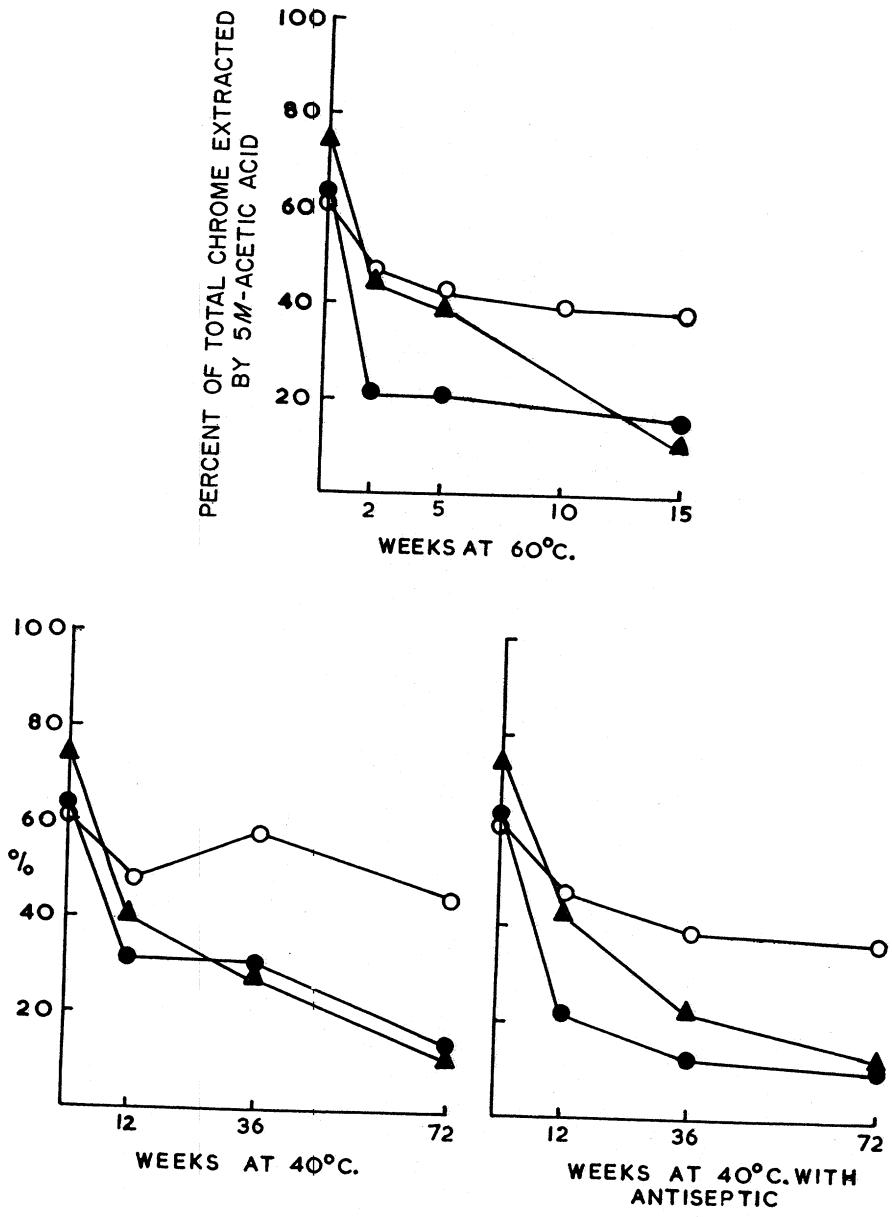
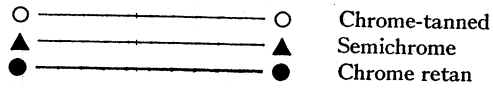


FIGURE 6.—Percentage of total chrome in leather extracted by 5*M* acetic acid.



storage it finally reached a steady value of about 40%. Even greater reductions in the chrome extracted were found with the semichrome and chrome retan leathers, less than 20% being soluble after the longest period of storage at either 60°C. or 40°C. (*see Fig. 6*), compared with 75% and 63% respectively in the original leathers.

DISCUSSION

The results of storage at 40°C. over water largely confirm those reported in Part I. The vegetable- and chrome-tanned leathers were fairly resistant to these conditions, but the combination-tanned leathers, although containing the same tans as the straight tannages, lost strength rapidly. The chrome-tanned leather was the only one on which the growth of mold had any observable effect. Kanagy *et al.* (4) also found relatively high losses in strength of a chrome leather on which mold had developed, but Roddy and Jansing (6) did not confirm this. It seems possible that there may be considerable differences between leathers or that particular molds are responsible. Further work is required to elucidate this behavior.

Although the general pattern of deterioration was the same at 60°C. as at 40°C., the relative behavior of the four types of leather was different. The chrome leather was still outstanding in its resistance, for although its strength was low after 15 weeks' storage, its appearance was still satisfactory. The vegetable-tanned leather, however, deteriorated rapidly, and after 10 weeks was more damaged than either of the combination-tanned leathers. This may be due to the fact that the temperature of storage was quite near the shrinkage temperature of the leather. Accelerated tests at 60°C. for the comparison of the stability of different types of leather are, therefore, not possible.

Some information regarding the breakdown of the protein can be deduced from the soluble nitrogen extracted. Hydrolysis of amide groups in the collagen would lead to increases in volatile nitrogen, but in many cases the amounts found exceed the maximum which would be expected from this source, i.e., about 2% of the total nitrogen. It appears, therefore, that deamination of amino acids must have occurred in some instances, particularly in the presence of mold. With the semichrome and retan leathers the losses of amino acids could amount to 5% of the total skin protein in the worst cases. No precautions were taken to avoid losses of volatile compounds during storage, but as the pH of the leathers was 4.0 or below, such losses seem unlikely. No appreciable change in nitrogen content of the leathers was detected.

The nonvolatile nitrogen probably consists mainly of peptides and amino acids. Increases did not necessarily follow losses in strength (*see also refs. 2, 3, and 14*). Relatively large losses in strength occurred without any appreciable increase in solubility, particularly with the chrome leather. High values were only obtained when the leathers were too weak to test, and their fiber structure was partially lost.

The glassy appearance of the middle layer of the leathers containing vegetable tan suggests that there may have been some degradation similar to the conversion of collagen to gelatin. Such changes in appearance did not occur at any stage with the chrome leather, and even under the microscope only slight evidence of damage could be seen. Other changes in the protein are not as extensive, and it seems likely that the mechanism of deterioration of this leather differs from that of leathers containing vegetable tans, in which oxidation and polymerization of the vegetable tan would also be expected to influence the deterioration.

The semichrome and chrome retan leathers behaved similarly to each other in general, suggesting that the order in which the two tanning processes were carried out was not important but that the presence of a chrome-vegetable tan complex was the controlling factor.

The effect of storage on the extractability of the chrome is difficult to explain. With the chrome leather it presumably indicates that the chrome complex had become more firmly fixed to the protein. This would also explain the increase observed in the shrinkage temperature. This explanation does not appear to apply to the combination-tanned leathers, in which the chrome tan was much less readily extracted, but the shrinkage temperature of the leather decreased. It seems likely that the chrome tan was removed from its complex with the collagen to form a more stable insoluble complex with the vegetable tan, as suggested in Part I. In the case of the retan leather stored for 15 weeks at 60°C., 5*M* acetic acid extracted 28.6% of the nitrogen of the leather, but only 15.8% of the chrome, suggesting that much of the chrome has been removed from the collagen and is present in a more insoluble complex.

It seems likely that the deterioration of the vegetable, semichrome, and retan leathers involves fairly extensive degradation of the protein. The results indicate that this may occur by at least two mechanisms, a hydrolytic breakdown of peptide chains and an oxidative mechanism probably involving deamination of the amino acids. In addition it is possible that there is a loss of molecular structure similar to the partial conversion of collagen to gelatin. With chrome leather the changes in the protein are small, perhaps because the few stable cross links formed are more effective in preventing disorganization of the protein molecule than the cross links in the other leathers.

Further information on the oxidative and hydrolytic breakdown of the protein in both leather and untanned skin is being sought.

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REFERENCES

1. Bowes, J. H., and Raistrick, A. S. Part I.
2. Kanagy, J. R., and Tobias, P. E. *JALCA*, **37**, 426 (1942).
3. ———, Charles, A. M., Abrams, E., and Tener, R. F. *JALCA*, **41**, 198 (1946).
4. ———, Seebold, R. E., Charles, A. M., and Cassell, J. M. *JALCA*, **44**, 270 (1949).
5. Lollar, R. M. *JALCA*, **49**, 605 (1954).
6. Roddy, W. T., and Jansing, J. J. *JALCA*, **49**, 773 (1954).
7. Mitton, R. G., and Turner, J. N. *J. Soc. Leather Trades Chemists*, **39**, 343 (1955).
8. Mann, C. W., and Steiner, E. T., quoted by Seligsberger, L., and Mann, C. W., in *Chemistry and Technology of Leather Manufacture* Vol. II (New York: Rheinhold, 1958) 489.
9. Seligsberger, L., Mann, C. W., and Clayton, H. *JALCA*, **53**, 627 (1958).
10. Mitton, R. G., and Wyatt, K. E. Unpublished work.
11. Sykes, R. L., and Williams-Wynn, D. *J. Soc. Leather Trades Chemists*, **43**, 265 (1959).
12. *Official Methods of Analysis*. Society of Leather Trades Chemists, Croydon, 1957.
13. Chibnall, A. C., Rees, M. W., and Williams, E. F. *Biochem. J.*, **37**, 354 (1943).
14. Mann, C. W., Steiner, E. T., Clayton, H., and Seligsberger L. *JALCA*, **54**, 680 (1959).